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Taura syndrome virus from Venezuela is a new genetic variant

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ABSTRACT

In early 2005, the Aquaculture Pathology Laboratory of the University of Arizona received samples of diseased shrimp, *Penaeus vannamei*, from a Taura syndrome (TS) outbreak in the Lake Maracaibo region of Venezuela. Histopathology and *in situ* hybridization (ISH) were performed and the results confirmed the presence of *Taura syndrome virus* (TSV). The viral isolate was sequenced and presented a 93% similarity with the TSV reference strain from Hawaii (TSV-HI94). Immunohistochemistry (IHC), dot blot immunoassay and bioassays were also performed. While processed samples reacted only faintly with the TSV monoclonal antibody MAb 1A1, the virus in its native state reacted strongly with the antibody. In bioassay, the Venezuelan isolate of TSV (TSV-VE05) presented mortality comparable to TSV-HI94 in *P. vannamei* SPF Kona stock. These data indicate that a new variant of the virus was responsible for the outbreak of TS in Venezuela.

1. Introduction

Since its first appearance in 1992, Taura syndrome (TS) has caused significant economic losses to the penaeid shrimp culture industry worldwide. The first TS epizootic was reported in Ecuador in 1992 (Jimenez, 1992). Outbreaks have since been reported from most shrimp farming regions of the America and Asia (Hasson et al., 1999a; Yu and Song, 2000; Nielsen et al., 2005; Do et al., 2006). This disease is known to affect many penaeid shrimp species. It has no known impact on the naupliar through mysis stages. However, it seriously affects the post-larvae, juvenile and adult stages of *Penaeus vannamei*, the most susceptible host (Brock, 1997).

The clinical signs of acute TS include anorexia, lethargy, erratic swimming behavior and expansion of red chromatophores (Lightner et al., 1995; Hasson et al., 1999b). This phase is followed by a transition phase, characterized by the presence of multiple melanized lesions on the cuticle of the tail and the cephalothorax, and a definitive chronic phase during which animals do not display any outward signs of infection. The chronic phase is characterized histologically by the presence of numerous lymphoid organ spheroids (LOS) in the absence of other pathology (Hasson et al., 1999c).

TS is caused by *Taura syndrome virus* (TSV) a member of the *Dicistroviridae* family (Mari et al., 2002; Mayo and Ball 2006). TSV is a non-enveloped, single-stranded positive sense RNA virus (Hasson et al., 1995; Bonami et al., 1997). The viral genome has been completely sequenced and consists of 10 205 nt encoding two open reading frames (ORF's). The 5' ORF (ORF1) encodes the non-structural proteins: a helicase, a protease and a RNA-dependent RNA polymerase. ORF2 encodes for 3 major and 1 minor capsid proteins (CP) of 40 kDa (CP1), 55 kDa (CP2), 24 kDa (CP3), and 58 kDa (Bonami et al., 1997; Mari et al., 2002).

Comparison of genome sequences has allowed the categorization of emerging TSV isolates into different genotypic clades. Phylogenetic analysis of TSV isolates from 1993 to 2004 revealed three major clusters designated as the "Americas", "Belize" and "SE Asia" (Tang and Lightner, 2005). TSV variants have also been identified according to the reaction to the TSV monoclonal antibody: MAb 1A1 (Erickson et al., 2003). MAb 1A1 is the only monoclonal antibody currently available for the antibody-based classification of TSV isolates. It has been shown to react to the 55 kDa CP2 (Poulos et al., 1999). The variants TSV-A (HI94 reference strain), TSV-B (MX98) and TSV-C (BZ02) are currently recognized (Erickson et al., 2003). Of these, TSV-A is the only variant reacting with MAb 1A1.

In 2005, the Aquaculture Pathology Laboratory (APL) received samples of diseased *P. vannamei* from an apparent TS outbreak in Venezuela. This represented the first recognized outbreak of TSV infection in farmed *P. vannamei* in Venezuela (Conroy, 2005). This isolate (hereafter referred to as TSV-VE05) did not show positive reaction by IHC with MAb 1A1. The genome was sequenced and compared to the Hawaiian reference strain (TSV-HI). The CP2 sequence was compared against other isolates collected during 1993–2005 (Tang and Lightner, 2005). The virulence of this isolate was evaluated in a bioassay using specific pathogen free (SPF) *P. vannamei* Kona stock (White et al., 2002). The findings suggest that TSV Venezuela is a new variant of the virus.

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2. Materials and methods

2.1. Shrimp and TSV isolates samples

The taxonomy used for the penaeids in this paper is according to Holthuis (1980). Shrimp samples (*P. vannamei*) from several farms in northwest Venezuela displaying gross signs characteristic of a TSV infection were received at the APL for diagnosis. The samples of *P. vannamei* juveniles, subadults and adults were fixed in Davidson's alcohol–formalin–acetic acid (AFA) for histological examination (Bell and Lightner, 1988). A separate set of samples was fixed in 95% ethanol for RT-PCR analysis. Additional frozen material was received and used in infectivity and virulence studies with SPF *P. vannamei* Kona stock (White et al., 2002).

2.2. In situ hybridization (ISH) and Immunohistochemistry (IHC)

Shrimp samples from the original case and the experimental bioassay were fixed by injection of Davidson's AFA followed by immersion in 10 volumes of fixative. The samples were transferred to 70% ethanol after 24 h, followed by a second transfer after 48 h. They were processed for routine paraffin embedding with Paraplast X-tra (McCormick Scientific), sectioning and staining with conventional Mayer–Bennett's hematoxylin/eosin–phloxin as described by Lightner (1996). Selected samples were evaluated by ISH with a digoxigenin-labeled gene probes P15/Q1 as described by Mari et al. (1998). IHC was performed using either MAb 1A1 specific for the detection of the original strain of TSV (TSV-HI94) (Poulos et al., 1999) or a polyclonal antibody (PAbs) as described byPoulos et al. (2001). The Mab 1A1 consisted of hybridoma supernatant fluid purified by anionic exchange and diluted 1:5 in blocking buffer (PBS with 2% powdered skim milk and 10% normal goat serum). The PAbs consisted of serum from mice immunized with TSV-BZ02 purified viral particles diluted 1:100 in blocking buffer. The PAbs is known to react with TSV HI94 and TSV BZ02 in a dotblot immunoassay and in IHC (Manuscript in preparation).

2.3. TSV RT-PCR and genomic sequencing

Total RNA was extracted from the ethanol fixed tissue samples using a High Pure RNA extraction kit (Roche Biochemical) according to the manufacturer's recommendations. Primers were designed using Primer3 (http://biotools.umas smed.edu/bioapps/primer3_www.cgi) and used to amplify the Venezuela TSV genome. SuperScript II one-step RT-PCR with Platinum Taq (Invitrogen) was used for the amplification. The cycling parameters consisted of 1 cycle at 50 °C for 30 min, 94 °C for 2 min and 39 cycles at 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1.5 min with a final extension cycle of 68 °C for 5 min. The RT-PCR products were visualized by electrophoresis of ethidium bromide stained agarose gels under UV light. The resulting bands were excised with a scalpel blade, cleaned with a QIAQuick PCR purification kit (Quiagen) and sequenced with a DNA sequencer ABI Prism 377 (Applied Biosystems) by the sequencing facility at the University of Arizona. The 10 095 nt sequence is available in GenBank under the accession number: DQ212790.

2.4. Phylogenetic analysis

The nt 7952-9151 sequence corresponding to the region encoding CP2 was compared against the TSV isolates from the UAZAPL collection (Table 1). The amino acid sequence was deduced from the nucleic acid sequence and the phylogenetic analysis was performed using the MEGA software package (http://www.megasoftware.net). The phylogenetic tree was constructed with the neighbor-joining (NJ) method (Saitou and Nei, 1987). The deduced amino acid sequences from the protein CP2 from Venezuela TSV and Hawaii TSV were further analyzed using the ProtParam open source software (http://ca.expasy.org/tools/protparam.html).

Table 1
Year of collection and origin of the TSV isolates collected from *Penaeus spp*

TSV isolate	Year	Origin	TSV isolate	Year	Origin
93EC	1993	Ecuador	04ER[b,9]	2004	Eritrea
94US — HI[1]	1994	US — Hawaii	04CH	2004	China
94EC	1994	Ecuador	05MX1	2005	Mexico
98MX[2]	1998	Mexico	05MX2	2005	Mexico
99MX1[a,3]	1999	Mexico	05MX3	2005	Mexico
99MX2[a,4]	1999	Mexico	05CH	2005	China
99TW[5]	1999	Taiwan	05VE	2005	Venezuela
2KMX[a,6]	2000	Mexico	05AW	2005	Aruba
01BZ[7]	2001	Belize	05ID	2005	Indonesia
02BZ	2002	Belize	05BZ1	2005	Belize
04TH1	2004	Thailand	05BZ2	2005	Belize
04TH2[b,8]	2004	Thailand	06ID	2006	Indonesia
04US — TX	2004	US — Texas	06AW	2006	Aruba
04MX	2004	Mexico	06-1	2006	Not specified
04BZ1	2004	Belize	06-2	2006	Not specified
04BZ2	2004	Belize	06MX	2006	Mexico
04BZ3	2004	Belize	06[TH]	2006	Thailand

[a] *P. stylirostris*. [b] *P. monodon*. Not labeled: *P. vannamei*. GenBank no. [1]AF277675, [2]AF510515, [3]AF510516, [4]AF277378, [5]AF406789, [6]AF510517, [7]AY590471, [8]DQ000306, [9]DQ000302.

2.5. Virus purification

Virus purification followed the technique described by Bonami et al. (1997) with minor modifications. Frozen shrimp heads were homogenized 1:3 in TN buffer (20 mM Tris, 0.4 M NaCl) using an Ultra-turrax tissue blender and clarified three times (10 min at 1000 g using an IEC HN-SII centrifuge followed by 15 min at 4300 g and 30 min at 30 600 g using an SS34 rotor in a Sorvall RC5-B Superspeed centrifuge). The pooled supernatant fluid was centrifuged at 205 100 g for 3 h (T647.5 rotor in a Sorvall Ultracentrifuge). The pellet was resuspended, homogenized and extracted 3 times with freon. Charcoal was added for 5 min and the supernatant fluid was filtered through Celite535 (Fluka) under vacuum and pelleted at 205 100 g for 3 h (T647.5 rotor). The pellet was homogenized and layered onto a 15–40% sucrose gradient and centrifuged at 233 000 g for 2 h (TH641 rotor). The gradient fractions were removed with an Autodensiflow IIC (Buchler) and collected using an ISCO Retriever II. The absorbance readings of the fraction were plotted upon collection by an ISCO UA5 monitor at a wavelength of 254 nm. The fractions associated with the peak were washed in TN and pelleted (233 000 g, 3 h, TH641 rotor). The pellet was homogenized in TN buffer and layered onto a 15–45% cesium chloride gradient (12 h, 208 500 g, TH641 rotor). The fractions were recovered as described above. Fractions associated with the peak were pooled, diluted with TN and pelleted at 233 000 g for 3 h (TH641 rotor). The final virus preparation was negatively stained with 2% phosphotungstic acid (PTA) at pH 7 on 200 mesh collodion carbon-coated grids and examined using a JEM-100CXII microscope (JEOL).

2.6. Dot blot immunoassay

The dot blot immunoassay was performed using MANAH45 multiscreen plate (Millipore). Purified TSV-VE05 virus preparations, native or boiled for 5 min, were dotted on the plate. The wells were blocked using 150 µl of blocking buffer for 30 min. The buffer was poured off and 100 µl of MAb 1A1 (1:5 in blocking buffer) or 100 µl of a PAb dilution (1:100 in PBS) was added to the wells and incubated for 30 min. The antibodies were poured off and washed 3 times with PBS before adding alkaline phosphatase labeled goat anti-mouse IgG F(ab')$_2$ heavy and light chains (Kierkegaar and Perry) as secondary antibody (30 min incubation with 100 µl of a 1:500 dilution). The supernatant fluid was poured off and the plate washed 3 times with PBS. The wells were developed using 100 µl of developing solution: